

COPY

Attorney Docket No. 0929.003/31807

**IN THE UNITED STATES
PATENT AND TRADEMARK OFFICE**

**RECEIVED
CENTRAL FAX CENTER**

JUL 29 2004

To the Application of:

de Boer, et al.

Serial No.: 08/070,158

Filed: May 28, 1993

For: "Anti-CD40 Monoclonal
Antibodies Capable of
Blocking B-Cell Activation"
(As Amended)

Group Art Unit: 1806

Examiner: Hutzell, P.

CERTIFICATE OF MAILING

I hereby certify that this paper is being
deposited with the United States Postal
Service as first class mail, postage
prepaid, in an envelope addressed to:
Hon. Commissioner of Patents and
Trademarks, Washington, D.C. 20231,
on this date:

1-20-95



Donald J. Pochopien
Registration No. 32,167
Attorney for Applicant

OFFICIAL

**DECLARATION OF MARK DE BOER
UNDER 37 C.F.R. §1.132**

Hon. Commissioner of Patents and Trademarks
Washington, D.C. 20231

Sir:

I, Mark de Boer declare as follows:

1. That I am a co-inventor of the above-identified application along with Leah B. Conroy.

2. That I am a co-author of the publication, de Boer *et al.*, "Generation of Monoclonal Antibodies To Human Lymphocyte Cell Surface Antigens Using Insect Cell Expressing Recombinant Proteins," *J. of Immunological Methods*, 152 15-23 (1992) (hereinafter "the de Boer publication").
3. That the de Boer publication discloses the anti-CD40 monoclonal antibodies and hybridomas of the above identified patent application, including 5D12, 3C6, and 3A8.
4. That the de Boer publication lists as co-authors Leah Controy, Hye Yeong Min and Jaap Kwekkeboom.
5. That the co-authors Hye Yeong Min and Jaap Kwekkeboom are not co-inventors of the subject matter disclosed in the above identified application, including the anti-CD40 monoclonal antibodies, such as 5D12, 3C6, and 3A8, or the hybridomas producing the same.
6. That Hye Yeong Min provided technical support but did not make an inventive contribution to the subject matter of the above identified application.
7. That Jaap Kwekkeboom provided technical support but did not make an inventive contribution to the subject matter of the above identified application.
8. That it is standard practice in the scientific community that publications, such as the de Boer publication, acknowledge the names of all persons who made technical contributions to the data presented.
9. That I am also a co-author of the publication, Kwekkeboom *et al.*, "CD40 Plays An Essential Role In The Activation Of Human B Cells By Murine BLA85 Cells," *Immunology*, 79 439-444 (1993) (hereinafter "the Kwekkeboom publication.")
10. That the Kwekkeboom publication lists as my co-authors J. Kwekkeboom, J.M. Tager and C. de Groot, none of whom are named as co-inventors of the above-identified application.
11. That the Kwekkeboom publication at page 440, under the heading "Antibodies and hCD40.H μ fusion protein" cites to its reference "8," *i.e.*, the de Boer publication of paragraph 2 herein, as the source of the anti-CD40 monoclonal antibodies 5D12, 3C6, 3A8, that are used therein, stating:

Anti-CD40 mAb 5D12, 3C6, and 3A8 were generated by immunizing mice with insect cells expressing recombinant CD40⁸.

12. That my co-authors of the Kwekkeboom publication, i.e., Kwekkeboom, Tager and de Groot, are not co-inventors of the anti-CD40 monoclonal antibodies of the present application, or the hybridomas producing these antibodies, or the methods of using these antibodies as disclosed in the specification of the above identified application.
13. That by citing to de Boer *et al.*, as reference "8" of Kwekkeboom, my co-authors on Kwekkeboom (i.e., Kwekkeboom, Tager and de Groot) tacitly admit that they are not co-inventors of the anti-CD40 monoclonal antibodies of the present application or the hybridomas for producing these antibodies.
14. That all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the above-identified patent application or any patent issuing thereon.


Mark de Boer

Dated: January 19, 1995

PATENT

Attorney Docket No. 0925.003/31807

**IN THE UNITED STATES PATENT
AND TRADEMARK OFFICE**

Applicant:
deBoer et al.

Serial No.: 08/070,158

Filed: May 28, 1993

**For: "Methods Of Blocking B-
Cell Activation Using Anti-
CD40 Monoclonal
Antibodies"**

Group Art Unit: 1806

Examiner: Phillip Gambel, Ph.D.

CERTIFICATE OF FACSIMILE

I hereby certify that this paper is being
sent via facsimile to: Assistant
Commissioner of Patents, Washington, DC
20231, attention Examiner Phillip Gambel,
Ph.D. at 703-308-4242 on this date:

11-20-96



Donald J. Pochoplen, Ph.D.
Reg. No. 32,167
Attorney for Applicant(s)

DECLARATION OF PAUL B. SAVEREIDE, Ph.D.

Assistant Commissioner of Patents
Washington, DC 20231

Sir:

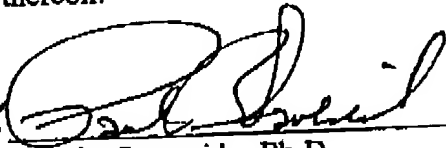
I, PAUL B. SAVEREIDE, declare that:

1. My title at Chiron Corporation is Assistant Secretary and Patent Counsel and in that capacity, I am authorized to act on behalf of Chiron Corporation in executing this document.
2. Chiron Corporation is an assignee of the above-identified patent application as reflected in an assignment recorded with the U.S. Patent and Trademark Office on January 26, 1995 at Reel 7329, Frames 0191-0195.
3. Hybridomas 5D12 and 3C6 were deposited with the American Type Culture collection, 12301 Parklawn Drive, Rockville, Maryland 20852, on May 6, 1993, and were given ATCC Accession Nos. HB 11339 and HB 11340, respectively.

Hybridoma 3A8 was deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852, on January 30, 1996, and was given ATCC Accession No. HB 12024.

4. In accordance with the "Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure," and as per the attached contract with the ATCC, all restrictions on the availability of hybridomas 5D12 (ATCC No. HB 11339), 3C6 (ATCC No. HB 11340) and 3A8 (ATCC No. HB 12024) will be irrevocably removed and these hybridomas will be made permanently available to anyone requesting said hybridomas upon the allowance of the above-identified patent application and the issuance of the patent thereon.
5. If the hybridomas of paragraph 3 should die or be destroyed during the effective term of the deposit, they shall be replaced with cultures of the same.
6. The biological materials deposited as per paragraph 3 above are identical to the biological materials specifically identified in the specification as filed.
7. I further declare that all statements made herein of my own knowledge are true, that all statements made on information and belief are believed to be true, and that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both (18 U.S.C. 1001), and may jeopardize the validity of the application or any patent issuing thereon.

Dated: November 19, 1996


Paul B. Savereide, Ph.D.
Registration No. 36,914

CHIRON CORPORATION
Intellectual Property-R440
4560 Horton Street
Emeryville, California 94608-2916
Phone: (510)923-2585
Facsimile: (510)655-3542



American Type Culture Collection

12301 Parklawn Drive • Rockville, MD 20852 USA • Telephone: (301)231-5520 Telex: 196455 ATCCNORTH • FAX: 301-770-2547

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT ISSUED PURSUANT TO RULE 7.3 AND VIABILITY STATEMENT ISSUED PURSUANT TO RULE 10.2

To: (Name and Address of Depositor or Attorney)

Chiron Corporation
Attention: Karen Van Note
4560 Horton Street
Emeryville, CA 94608-2916

Deposited on Behalf of: Chiron Corporation (Case No. #2630.2)

Identification Reference by Depositor:

ATCC Designation

Hybridoma 5D12, CMCC 11068
Hybridoma 3C6, CMCC 11067
Hybridoma B7-24-E1G4, CMCC 11060

HB 11339
HB 11340
HB 11341

The deposits were accompanied by: ☐ a scientific description ☐ a proposed taxonomic description
Indicated above.

The deposits were received May 6, 1993 by this International Depository Authority and have been
accepted.

AT YOUR REQUEST:

☒ We will inform you of requests for the strains for 30 years.

The strains will be made available if a patent office signatory to the Budapest Treaty certifies one's
right to receive, or if a U.S. Patent is issued citing the strains.

If the cultures should die or be destroyed during the effective term of the deposit, it shall be your
responsibility to replace them with living cultures of the same.

The strains will be maintained for a period of at least 30 years after the date of deposit, and for a
period of at least five years after the most recent request for a sample. The United States and many
other countries are signatory to the Budapest Treaty.

The viability of the cultures cited above was tested May 13, 1993. On that date, the cultures were
viable.

International Depository Authority: American Type Culture Collection, Rockville, Md. 20852 USA

Signature of person having authority to represent ATCC:

Bobbie A. Brandon
Bobbie A. Brandon, Head, ATCC Patent Depository

Date: May 21, 1993

cc: Mr. Kenneth Goldman



American Type Culture Collection

12301 Parklawn Drive • Rockville, MD 20852 USA • Telephone: (301)231-5520 Telex: 908-768 ATCCRUVE • FAX: 301-816-4346

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF
THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT ISSUED PURSUANT TO RULE 7.3
AND VIABILITY STATEMENT ISSUED PURSUANT TO RULE 10.2

To: (Name and Address of Depositor or Attorney)

Chiron Corporation
Attention: Karen Van Note
4580 Horton Street
Emeryville, CA 94608-2918

Deposited on Behalf of: Chiron Corporation (Case No. 0925.003)

Identification Reference by Depositor:	ATCC Designation
Hybridoma, 3A8, CMCC #11088	HB 12024

The deposit was accompanied by: ☐ a scientific description ☐ a proposed taxonomic description indicated above.

The deposit was received January 24, 1996 by this International Depository Authority and has been accepted.

AT YOUR REQUEST:

☒ We will inform you of requests for the strain for 30 years.

The strain will be made available if a patent office signatory to the Budapest Treaty certifies one's right to receive, or if a U.S. Patent is issued citing the strain, and ATCC is instructed by the United States Patent & Trademark Office or the depositor to release said strain.

If the culture should die or be destroyed during the effective term of the deposit, it shall be your responsibility to replace it with living culture of the same.

The strain will be maintained for a period of at least 30 years after the date of deposit, and for a period of at least five years after the most recent request for a sample. The United States and many other countries are signatory to the Budapest Treaty.

The viability of the culture cited above was tested January 30, 1996. On that date, the culture was viable.

International Depository Authority: American Type Culture Collection, Rockville, Md. 20852 USA

Signature of person having authority to represent ATCC:

Barbara M. Halley
Barbara M. Halley, Administrator, ATCC Patent Depository

Date: February 6, 1996

✓cc: Paul Saveriede



☐ EPA/EPO/OEB
D-80285 München
+ 49 89 2399-0
TX 523 656 apm u d
FAX + 49 89 2399-4405

Europäisches
Patentamt

European
Patent Office

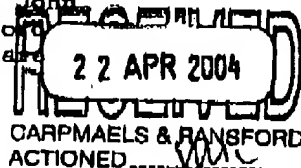
Office européen
des brevets

Generaldirektion 2

Directorate General 2

Direction Générale 2

Marshall, Cameron John
Carpmaels & Ransford
43 Bloomsbury Square
London WC1A 2RA
GRANDE BRETAGNE



COPY

Datum/Date

20.04.04

Zektion/Ref./Ref. N. 73464A TJD/cm	Anmeldung Nr./Application No./Demande n°/Patent Nr./Patent No./Brevet n° 99104709.3-2401/0945465
Anmelder/Applicant/Demandeur/Proprietor/Théâle CHIRON CORPORATION	

COMMUNICATION PURSUANT TO ARTICLE 115(2) EPC

Please find enclosed observations by a third party concerning the patentability of the invention of the above-mentioned patent application. That person is not a party to the proceedings before the EPO (Art. 115(1) EPC).

Under Article 115(2) EPC you may comment on the observations.

Formalities Officer
Tel. No. 089/2399 - 7548

A. Raup

Raup

HOFFMANN · EITL

MÜNCHEN LONDON

HOFFMANN · EITL · Postfach 81 04 20 · D-81904 München

FACSIMILE 089/2399-8787

European Patent Office

80298 Munich

**EPO - Munich
12**

29. März 2004

Munich, March 29, 2004

Our Ref.: AV-2004-264 m2

EP 99104709.3 In the name of Chiron Co. entitled "Antagonistic monoclonal antibodies to human CD40"

*Please find enclosed third party observations pursuant to
Article 115 EPC.*

1. Present claims

In their letter dated 27 January, 2004, the patentee submitted an amended set of claims 1-13.

Claim 1 recites a monoclonal antibody or fragment thereof capable of binding to a human CD40 antigen located on the surface of a human B cell, wherein the binding of the antibody or fragment thereof to the CD40 antigen prevents the growth or differentiation of the B cell.

Claim 2 is directed to the specific monoclonal antibodies 5D12 and 3C6. Claims 3 and 4 are dependent on claim 2 which recites these specific antibodies.

Claim 5 relates to a hybridoma capable of producing a monoclonal antibody having specificity for the CD40 antigen of a human B cell,

PATENTANWÄLTE · MÜNCHEN EUROPEAN PATENT ATTORNEYS EUROPEAN TRADEMARK ATTORNEYS

Klaus Fuchsle, Dipl.-Ing.
Bernd Hansen, Dr., Dipl.-Chem.
Klaus Öberg, Dipl.-Ing.
Karl Kohlmann, Dipl.-Ing.
Bernhard von Hirsch, Dipl.-Ing.
Rainer Zangs, Dipl.-Ing.
Matthias Kündler, Dr., Dipl.-Chem.
Roy D. Marsh, M.A. (Oxon) C.P.A. (I)
Christiane Stein-Dodger, Dr., Dipl.-Chem.
Detlev A. Post, Dr., Dipl.-Phys.
Velt Frank, Dipl.-Ing., M.Sc. (London)
Leo Polz, Dr., Dipl.-Chem.
Christoph Furbach, B.E. (U.C.D.) C.P.A. (I)
Thorsten Bausch, Dr., Dipl.-Chem.
Thomas Koch, Ph.D. (London), Dipl.-Ing.
Friedrich Heber, Dipl.-Phys. (I)
Joseph Taumilho, Dr. (J.F.U.) (I)
Peter Klusmann, Dr., Dipl.-Chem.
Georg Singer, Dr., Dipl.-Phys.
Peter Schweighart, Dr., Dipl.-Ing.
Stefan Mayrhofer, Dr., Dipl.-Ing.
Matthias Wolf, Dr., Dipl.-Chem.
Klemens Seitzmann, Dr., Dipl.-Chem.
Peter Wiedemann, Dipl.-Ing.
Sabine Böhm, M.D. (London), B.Sc.
Lorenz von Kurowski, Dr., Dipl.-Ing.
Joachim Renken, Dr., Dipl.-Chem.
Jan-Flendrik Spillies, Dr., Dipl.-Chem.
C. Thomas Becker, Dipl.-Ing.
Elisabeth Engelhard, Dr., Dipl.-Dipl.
Martin Bachstein, Dr., Dipl.-Chem.
Georg M. Kreuz, Dipl.-Ing. (I)

RECHTSANWÄLTE · MÜNCHEN ATTORNEYS AT LAW EUROPEAN TRADEMARK ATTORNEYS

Günther Rahn, Dr. jur.
Wolfgang v.d. Osten-Sacken, LL.M.
Holger Strammann
Alexandros Sprenger
Anja Petersen-Padberg, Dr. jur.
Alexander González, Dr. jur.
Christian Holger Foltz, Dr. jur.
Matthias Keespies, Dr. jur., LL.M.

BRITISH PATENT ATTORNEYS · LONDON EUROPEAN PATENT ATTORNEYS EUROPEAN TRADEMARK ATTORNEYS

Stephen J. Avery, M.A. (Oxon)
David J. Lethem, M.A. (Oxon) (I)
Alan Mitchell, M.A. (Oxon) (I)
Susan M. Dupuy, M.Sc. (London)

CONSULTANTS · MÜNCHEN PATENTANWÄLTE · RECHTSANWÄLTE EUROPEAN PATENT ATTORNEYS ATTORNEYS AT LAW

Werner Eitel, Dipl.-Ing.
Klaus Hoffmann, Dr., Dipl.-Ing.
Werner Lehn, Dipl.-Ing.
Alexander Netze

(I) nicht Patentbewerkstelligter German Patent Attorney
(I) nicht Patentbewerkstelligter European Patent Attorney
(I) nicht Patentbewerkstelligter European Trademark Attorney

MÜNCHEN

Arabellastrasse 4
D-81925 München
pm@hoffmann-eitel.com
www.hoffmann-eitel.com
Telefon +49 (0)89-92409-0
Fax Munich +49 (0)89-6816356

LONDON

Sardinia House,
Sardinia Street
52 Lincoln's Inn Fields
London WC2A 3LZ
Fax London +44 (0)20 7638 9218
Phone London +44 (0)20 7638 9218

wherein the binding of said monoclonal antibody to a human CD40 antigen expressed on the surface of a human B cell inhibits the growth or differentiation of the B cell.

Claims 6 and 7 recite the same specific antibodies of claim 2.

Claim 10 relates to a CD-40 antigen epitope immunoreactive with an anti-CD-40 monoclonal antibody.

Claims 8, 9 and 11-13 relate to 2nd medical indications using the above monoclonal antibodies or pharmaceuticals comprising these antibodies.

II. The pending claims cannot enjoy the 1st priority date

D1: Journal of Immunological Methods, vol. 152, no. 1, 31 July 1992 (1992-07-31), pages 15-23. (Cited International search report and EPO search report)

In the priority application USSN 07/910222 (filed on July 9, 1992), there is no description regarding the specific feature of the anti-CD40 antibody recited in claims 1 and 5, the specific use of the anti-CD40 antibody recited in claims 8, 9 and 11, the pharmaceutical composition recited in claims 12 and 13, and the specific CD40 antigen epitope recited in claim 10. That is, USSN 07/910222 never discloses an antibody which can prevent the growth or differentiation of B cells.

Regarding the hybridoma claims (claims 5 to 7) and the claim reciting specific antibody clones (claim 2), there is no description of depository number of hybridomas producing monoclonal antibodies 5D12 and 3C6 in USSN 07/910222. Based on the description on page 81 of the present specification, the depository date of hybridoma 3C6 and 5D12 is May 6, 1993. The claimed invention cannot be enabled without the deposition of hybridomas.

Therefore, the effective application date which is the date to judge the novelty and inventive step is the international filing date, July 8, 1993, or at the earliest, May 28, 1993 (filing date of 3rd priority application).

III. Lack of patentability over de Boer et al (D1)

D1, which was published before May 28, 1993, discloses the anti-CD40 antibodies termed 3A8, 3C6, 5D12 and 5H7 and hybridomas producing these antibodies. D1 also describes that these monoclonal antibodies can be bound to CD40 on B cells (see page 21, left column, lines 12-15; Table 11). CD40 antibodies with the same names and hybridomas producing these same antibodies are described in the present specification.

Present claims 2, 6 and 7 are not novel over D1 since the claimed antibodies and hybridomas are indistinguishable from those disclosed in D1. Claims 1 and 5 also lack novelty over D1, since claims 1 and 5 comprise 5D12 and 3C6 antibodies and hybridomas, respectively. Claims 3 and 4 are anticipated by D1, since humanized antibody and antibody fragments such as Fab' are well-known variations of the monoclonal antibody

At the time of filing the present application, testing B cell proliferation activity was a routine experiment for characterization of the anti-CD40 antibody. Therefore, the person skilled in the art can readily find the specific feature of the 5D12 and 3C6 antibodies, described in D1. Therefore, claims 8, 9, 11, 12 and 13 are readily conceived based on the descriptions of D1.

Regarding claim 10, the third party observer would like to point out that no specific CD40 antigen epitope is shown in the specification.



Joseph Taormino, Ph.D.
Association No. 151

Encl.:
D1: Journal of Immunological Methods, vol. 152, no. 1, 31 July 1992

D 1

Vol. 152, no. 1, 1992

ISSN 0022-1759

JIMMBG 152(1) 1-148 (1992)

Dsp. van Ned

5 AUG 1992

Publication Kit

JOURNAL OF

IMMUNOLOGICAL METHODS

EDITORS:

V. NUSSENZWEIG

M.W. TURNER



ELSEVIER

P.02

TO 00012026725399

18:19 FROM KON B18

2004/08/02

P.017

TEL:9198622260

JUL - 29 04 (THU) 17:43

JIM 06150

Generation of monoclonal antibodies to human lymphocyte cell surface antigens using insect cells expressing recombinant proteins

Mark de Boer ^a, Leah Controy ^b, Hye Yeong Min ^a and Jaap Kwakkeboom ^a

^a Departments of Immunology, and ^b Molecular Biology, Cetus Corporation, Emeryville, CA, USA, and ^c Department of Cell Biology and Histology, University of Amsterdam, Amsterdam, Netherlands

(Received 19 December 1991, revised received 11 February 1992, accepted 12 February 1992)

We have expressed human CD40 and human B7 in insect cells using the baculovirus expression system and have used these insect cells to immunize mice for the generation of monoclonal antibodies. We demonstrate here that specific monoclonal antibodies to human CD40 and human B7 were obtained using this approach. One significant advantage of this method is that immunizing mice with insect cells did not evoke an immune response to human cells and, therefore, EBV-transformed human B cells could be used to screen for specific antibody production by the hybridoma clones.

Key words: Baculovirus expression; Immunization; Monoclonal antibody; Lymphocyte cell surface antigen; Polymerase chain reaction

Introduction

Since the introduction of hybridoma technology by Köhler and Milstein (1975), a large number of monoclonal antibodies have been produced, which specifically bind to molecules expressed on the cell surface of lymphoid cells. These monoclonal antibodies have proven to be powerful tools in immunological research. To obtain monoclonal antibodies against cell surface molecules, mice have been immunized with the cells expressing the molecule of interest. Immunizing mice with whole cells usually results in a strong immune response and, therefore, the use

of adjuvants is generally not required. Instead, the question is more how to suppress the immune response to unwanted immunodominant antigens. When mice are immunized with whole cells, antibodies to a large number of different molecules are generated. It is therefore difficult to use the same cells to screen specific antibody production by the hybridoma clones. The cells used for immunization can be used for a pre-screen or in a differential screen, but additional screening assays are needed to determine the specificity of the monoclonal antibodies obtained. Furthermore, when the molecule of interest is expressed at low density, it is likely that the frequency of mouse B cells specific for the antigen will be very low in a strong polyclonal immune response. As a consequence, large numbers of hybridoma clones have to be screened for specific antibody production.

Correspondence to: M. de Boer, Innogenetics N.V., Industriepark Zwijnaarde 7, Box 4, B-9052 Ghent, Belgium. Fax: 31-91-410799.

To circumvent these problems, murine fibroblasts expressing human cell surface antigens have been used to immunize for specific antibody production (DiSanto et al., 1991). When injected into the appropriate mouse strain, the syngeneic fibroblasts should not be immunogenic and the immune response should be focused on the xenogeneic recombinant protein. Here we have used a slightly different approach to address the above-mentioned problems. We have expressed several human cell surface antigens in insect cells and used these insect cells to immunize mice. Using the baculovirus expression system (reviewed by Webb and Summers, 1990), cell surface proteins can be expressed at very high levels in insect cells (Webb et al., 1989). When these cells are used for immunization, the high expression levels of the cloned proteins will increase the chance of obtaining specific antibodies. We demonstrate that immunizing mice with insect cells expressing human CD40 or human B7 resulted in production of specific monoclonal antibodies to these molecules. Furthermore, immunization of mice with insect cells alone did not evoke an immune response to human cells and, therefore, human EBV-transformed B cells could be used for the screening of specific antibodies to human CD40 or human B7.

Materials and methods

Materials

Iscove's modification of Dulbecco's modified Eagle's medium (IMDM) and fetal bovine serum were obtained from JR Biosciences (Lenexa, KS); penicillin and streptomycin were obtained from Irvine (Santa Ana, CA); and polyethylene glycol (mol. wt. 1500) was obtained from Boehringer Mannheim (Indianapolis, IN).

Cell lines

SP2/0 murine myeloma cells, the EBV-transformed B cell line ARC, the T cell lines HSB and CEMM, and Sf9 insect cells were all obtained from the ATCC (Rockville, MD).

Culture media

SP2/0 murine myeloma cells, hybridoma cells, and cell lines were cultured in IMDM supplemented

with streptomycin (200 µg/ml), penicillin (200 U/ml) and 10% heat-inactivated fetal bovine serum (complete IMDM). The Sf9 insect cells were cultured in shaker flasks agitated (125–150 rpm) in medium described by Maiorella et al. (1988) supplemented with 0.5% fetal bovine serum.

Antibodies

Anti-human B7 monoclonal antibody BB-1 (Yokochi et al., 1982) was a gift of Dr. E.A. Clark (University of Washington, Seattle, WA) and was used as purified antibody. Anti-human CD40 monoclonal antibody G28.5 (Clark and Ledbetter, 1986) was a gift of Dr. J.A. Ledbetter (Oncogen Corporation, Seattle, WA) and was used as purified antibody. Anti-CD40 monoclonal antibody S2C6 (Paulic et al., 1985) was a gift of Dr. S. Paulic (University of Stockholm, Stockholm, Sweden) and was used as purified antibody. Anti-human CD26 monoclonal antibody Ta-1 and anti-CD20 monoclonal antibody B1 were obtained from Coulter (Hialeah, FL). Anti-CD3 monoclonal antibody OKT3 was obtained from Ortho (Raritan, NJ), and the anti-LeuM3 monoclonal antibody was obtained from Becton-Dickinson (San Jose, CA). Anti-IgM antibodies coupled to beads (Immunobeads) were obtained from Bio-Rad (Richmond, CA). The monoclonal antibody to the Glu-Glu epitope (Grussenmyer et al., 1985) was used as purified antibody.

PCR cloning of CD40 and B7

RNA was isolated from a population of EBV-transformed human spleen cells as described by Chirgwin et al. (1979). In brief, the cells were washed twice with phosphate buffered saline and lysed in 5 M guanidinium thiocyanate in the presence of 0.2 M 2-mercaptoethanol. The cell lysate was layered on a discontinuous CsCl gradient and centrifuged for 16 h at 26,000 rpm in a Beckman SW28 rotor. The RNA was recovered by dissolving the pellet in diethyl pyrocarbonate-treated H₂O (DEPC treated H₂O). The RNA was precipitated with ethanol once, resuspended in DEPC-treated H₂O, and stored at –70°C.

Total RNA (10 µg/reaction) was converted to cDNA using random hexamer priming in 50 µl

JUL -29 04 (THU) 17:44

18

fied from concentrated SF9 insect cell supernatants by affinity chromatography. The affinity matrix consisted of protein G-Sepharose coupled with a monoclonal antibody to the Glu-Glu epitope. Concentrated insect cell supernatant was dialyzed against phosphate-buffered saline (PBS) and loaded onto the column. The column was washed with 5 column volumes of PBS + 0.2% octyl glucoside and 2 mM 2-mercaptoethanol, followed by 5 column volumes of elution buffer (50 mM NaBO₃, pH 9.0, 2 mM 2-mercaptoethanol, 0.2% octyl glucoside). The soluble B7 or soluble CD40 tagged with the Glu-Glu epitope was released from the column by adding free Glu-Glu peptide (50 µg/ml in elution buffer). The fractions were neutralized with 0.5 M acetic acid, 0.5 M sodium phosphate, pH 5.5. The eluted proteins were then dialyzed against PBS.

Immunization

Female BALB/c mice were injected intraperitoneally (IP) at day 0 and day 14 with 5×10^6 SF9 cells infected with AcCD40 virus, AcB7 virus, or AcCd3 virus (control virus). On day 21, 100 µl of serum was obtained to test for the presence of specific antibodies. After a rest period of at least two weeks, the mice received a final injection with 5×10^6 SF9 cells infected with AcCD40 or AcB7 virus. Three days after this last injection, the spleen cells were used for cell fusion.

Generation of hybridoma clones

Splenocytes from immunized BALB/c mice were fused with SP2/0 murine myeloma cells at a ratio of 10:1 using 50% polyethylene glycol as previously described by De Boer et al. (1988). The fused cells were resuspended in complete IMDM medium supplemented with hypoxanthine (0.1 mM), aminopterin (0.01 mM), thymidine (0.016 mM) and 0.5 ng/ml hIL-6 (Genzyme, Cambridge, MA). The fused cells were then distributed between the wells of 96-well tissue culture plates, so that each well contained one growing hybrid on average. After 10–14 days the supernatants of the hybridoma populations were screened for specific antibody production. For the screening of specific antibody production by the hybridoma clones, the supernatants of 12 wells were pooled and used for fluorescent cell

staining on EVB-transformed B cells as described below. Subsequently, the supernatants of the positive pools were tested individually. Positive hybridoma cells were cloned three times by limiting dilution in complete IMDM containing 0.5 ng/ml hIL-6.

SF9 cell ELISA

SF9 insect cells infected with recombinant virus were cultured for 48 h in 24-well plates. After removal of the tissue culture medium the plates were incubated for 45 min at room temperature (RT) with 0.25 ml of antibody in PBS with 1% BSA (PBS-BSA). After three washes with PBS-BSA, the plates were incubated for 45 min at RT with 250 µl of a 1/250 dilution of goat anti-mouse total Ig immunoglobulins conjugated to horseradish peroxidase (Zymed, South San Francisco, CA) in PBS-BSA. Unbound peroxidase activity was removed by washing five times with PBS-BSA. Bound peroxidase activity was revealed by the addition of an assay mixture prepared by diluting 0.5 ml of 2 mg/ml 3,3',5,5'-tetramethylbenzidine in ethanol to 10 ml with 10 mM Na acetate, 10 mM EDTA buffer (pH 5.0) and adding 0.03% (v/v) H₂O₂. The reaction was stopped after 10 min by adding 100 µl of 1 M H₂SO₄.

Isolation of tonsillar B cells

Tonsillar B lymphocytes were isolated from tonsils obtained from children undergoing tonsillectomy as described by De Groot et al. (1990). Briefly, the tissue was dispersed with scalpel blades, phagocytic cells and NK cells were depleted by treatment with 5 mM L-leucine methyl ester, and T cells were removed by one cycle of rosetting with sheep erythrocytes treated with 2-aminoethyl isothioluronium bromide.

Fluorescent cell staining

Cells (10^6 /sample) were incubated in 100 µl primary antibody (10 µg/ml in PBS-BSA or Hanks' balanced salt solution (HBSS) supplemented with 1% BSA and 0.05% sodium azide) for 20 min at 4°C. After three washes with PBS-BSA or HBSS-BSA, the cells were incubated in 100 µl FITC-labeled F(ab')₂ fragments of goat anti-mouse IgG antibodies (Jackson, West

19

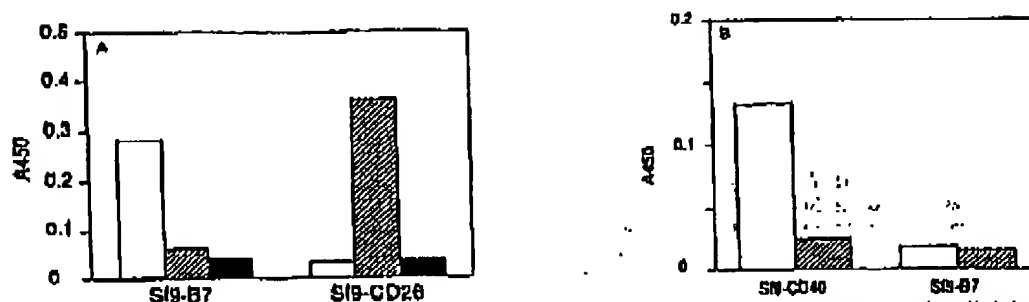


Fig. 2. ELISA assays on live SF9 cells were performed as described in the materials and methods section. A: SF9 cells infected with pAcB7 and pAcCD26 were cultured for 48 h in 24-well plates. The antibodies used in the ELISA were: B7-24, anti-B7 (open bars), Ta-1, anti-CD26 (hatched bars) and no primary antibody (gray bars). B: SF9 cells infected with pAcB7 and pAcCD40 were cultured for 48 h in 24-well plates. The antibodies used in the ELISA were: S2C6, anti-CD40 (open bars) and no primary antibody (hatched bars).

Grove, PA) for 20 min at 4°C. After three washes with PBS-BSA or HBSS-BSA and one wash with PBS, the cells were resuspended in 0.5 ml PBS. Analyses were performed with a FACSCAN V (Becton Dickinson, San Jose, CA). For analysis of cultured tonsillar B cells, propidium iodide was used to exclude dead cells.

Results

Full length cDNAs encoding human B7 and human CD40 were generated by PCR using

primers with restriction sites for cloning. The template for PCR amplification was cDNA generated from EBV-transformed human spleen B cell RNA. The expression of the recombinant molecules on the cell surface of the SF9 cells was tested using an ELISA system. Fig. 2a shows that the anti-B7 monoclonal antibody BB-1 reacted only with SF9 cells infected with AcB7 virus, but not with SF9 cells expressing human CD26. In contrast, the anti-CD26 monoclonal antibody Ta-1 reacted only with the SF9 cells expressing CD26, but not B7. Similar results were obtained with SF9 cells expressing CD40. Fig. 2b shows

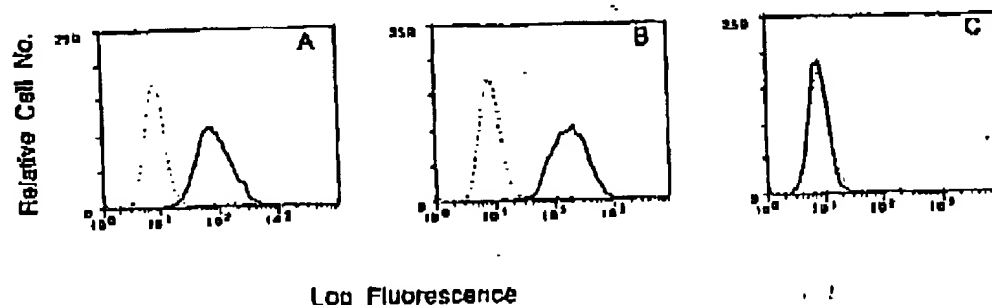


Fig. 3. Fluorescent cell staining of ARC cells as described in the materials and methods section. A: staining with a 1/100 dilution of serum from a mouse immunized with B7 expressing SF-9 cells (solid line) or a 1/100 dilution of normal mouse serum (dotted line). B: staining with a 1/100 dilution of serum from a mouse immunized with CD40 expressing SF9 cells (solid line) or a 1/100 dilution of normal mouse serum (dotted line). C: staining with a 1/100 dilution of serum from a mouse immunized with control SF9 cells (solid line) or a 1/100 dilution of normal mouse serum (dotted line).

that the anti-(CD40) monoclonal antibody 52C6 reacted only with Sf9 cells expressing CD40, but not with Sf9 cells expressing B7.

For the generation of monoclonal antibodies to CD40 and B7, mice were immunized with the Sf9 cells expressing these molecules on the cell surface. 1 week after the second immunization, the mice were bled and the sera were analyzed for the presence of specific antibodies using fluorescent cell staining of EBV-transformed B cells. Fig. 3 shows that mice immunized with Sf9 cells expressing CD40 or B7 had a serum titer against EBV-transformed B cell line ARC, which is positive for both CD40 and B7. In contrast, mice which were immunized with control Sf9 cells showed no reactivity with the ARC cells.

Since the mice immunized with Sf9 cells expressing CD40 or B7 had a serum titer when tested on ARC cells, we gave one mouse a final booster injection with CD40 expressing Sf9 cells and one with B7 expressing Sf9 cells. 3 days after the booster injection, the spleens were removed

TABLE I

SUMMARY OF FUSION DATA FOR THE GENERATION OF MONOCLONAL ANTIBODIES TO HUMAN CD40 AND HUMAN B7

Fusion	Anti-CD40	Anti-B7
no. of wells seeded after fusion	480 ^a	960
no. of wells with hybridoma growth	351	312
no. of positive wells ^b	4	1
Frequency of positive wells ^c	1.15	0.31

^a Only half of the cells obtained after fusion were analyzed.

^b As determined by FACS analysis described in the materials and methods section.

^c The frequency of positive wells is defined as the number of positive wells divided by the total number of wells with hybridoma growth, multiplied by 100.

and the splenocytes were fused with SP2/0 murine myeloma cells. Table I gives a summary of the fusion data. After the CD40 fusion, only half of the cells were seeded in 480 wells. This resulted in 351 wells with hybridoma growth. After

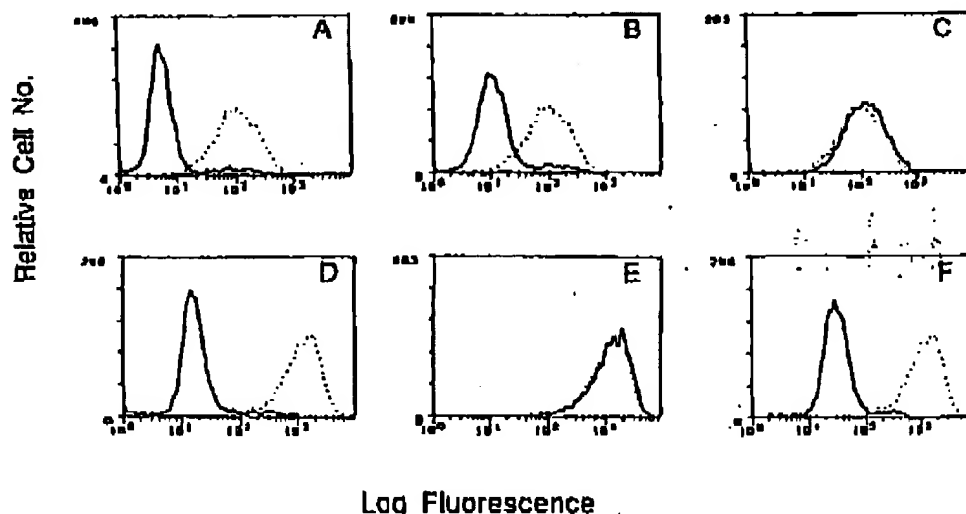


Fig. 4. Fluorescent cell staining of ARC EBV-transformed B cells. Staining with anti-(B7) and anti-(CD40) monoclonal antibodies in the presence and absence of soluble B7 and soluble CD40. The antibodies and the soluble B7, soluble CD40, or controls were preincubated at RT for 20 min before addition to the ARC cells. A: staining with B7-24 (dotted line) or secondary antibody only (solid line). B: staining with B7-24 alone (dotted line) or B7-24 preincubated with soluble B7 (solid line). C: staining with B7-24 alone (dotted line) or B7-24 preincubated with soluble CD40. D: staining with CD40-3A8 (dotted line) or second antibody only (solid line). E: staining with CD40-3A8 alone (dotted line) or CD40-3A8 preincubated with soluble B7 (solid line). F: staining with CD40-3A8 alone (dotted line) or preincubated with soluble CD40.

the B7 fusion, the cells were distributed in 960 wells and this fusion yielded 312 wells with hybridoma growth. 14 days after the fusions, supernatants of 12 wells were pooled and the pools were tested for the presence of antibodies reactive with ARC cells. FACS analysis revealed that four pools from the CD40 fusion and one pool from the B7 fusion were reactive with ARC cells. When individual supernatants from the positive pools were retested, four wells reactive with CD40 and one well reactive with B7 were identified. The cells from these positive wells were cloned by limiting dilution and, after three rounds, four stable anti-(CD40) hybridoma clones (CD40-3A8, CD40-3C6, CD40-5D12 and CD40-5H7) and 1 stable anti-(B7) hybridoma clone (B7-24) were established.

All five hybridoma clones were reactive with ARC cells and other EBV-transformed B cell lines, but not with T-cell lines HSB and CEMM (results not shown). However, the specificity of these monoclonal antibodies had yet to be proven. Therefore, we performed competition experiments in which soluble forms of CD40 and B7 competed with CD40 and B7 expressed on the ARC cells for the binding of the monoclonal antibodies. Hybridoma supernatants were pre-incubated with soluble forms of CD40 and B7. Subsequently, the mixtures were added to ARC cells for fluorescent cell staining. Fig. 4 shows that soluble B7, but not soluble CD40, could block the binding of anti-(B7) monoclonal antibody B7-24 to ARC cells. Conversely, soluble CD40, but not soluble B7, could block the binding of anti-(CD40) monoclonal antibody CD40-3A8 to ARC cells. Similar results were obtained with the other three anti-(CD40) monoclonal antibodies. Furthermore, the effects of soluble CD40 on the anti-(CD40) monoclonal antibodies and the effect of soluble B7 on the anti-(B7) monoclonal antibody was concentration-dependent. Decreasing the amount of soluble protein resulted in decreased blocking of antibody binding to ARC cells (results not shown).

For further analysis of the anti-(CD40) and anti-(B7) monoclonal antibodies, we tested their ability to bind to tonsillar B cells. Table II shows that 89-95% of freshly isolated tonsillar B cells stained positive with the four anti-(CD40) mono-

TABLE II
BINDING OF ANTI-(CD40) MONOCLONAL ANTIBODIES TO HIGHLY ENRICHED TONSILLAR B CELLS

Antibody	Specificity	Percentage of positive cells *
OKT3	CD3	2.1
LeuM3	LeuM3	1.5
B1	CD20	88.0
O28.5	CD40	92.1
CD40-5H7	CD40	93.7
CD40-5D12	CD40	95.0
CD40-3C6	CD40	88.9
CD40-3A8	CD40	93.3

* The percentage of positive tonsillar cells was measured by fluorescent cell staining as described in the materials and methods section.

clonal antibodies. About the same percentage of cells was positive with anti-(CD40) monoclonal antibody O28.5. Table III shows that 12-17% of freshly isolated tonsillar B cells stained positive with anti-(B7) monoclonal antibody B7-24. However, when tonsillar B cells were cultured for 5 days in the presence of immobilized anti-(IgM) antibodies and IL-2, the percentage of cells positive for B7-24 increased up to about 25% (results not shown). Furthermore, when tonsillar B cells were stimulated with anti-(IgM) antibodies and IL-2, not only did the number of B cells positive for B7-24 increase, but there was also a significant increase in the amount of fluorescent staining per cell, indicating that the expression of B7 was increased after stimulation (results not shown).

TABLE III
BINDING OF ANTI-(B7) MONOCLONAL ANTIBODY B7-24 TO HIGHLY ENRICHED TONSILLAR B CELLS

Antibody	Specificity	Percentage of positive cells *	
		Donor 1	Donor 2
OKT3	CD3	8.0	2.1
B1	CD20	74.0	88.0
B7-24	B7	12.6	16.8

* The percentage of positive tonsillar cells was measured by fluorescent cell staining as described in the materials and methods section.

Discussion

For the production of monoclonal antibodies one would generally like to immunize mice with purified material. However, purification of membrane antigens requires specialized and complex techniques and, furthermore, extraction from the membrane may alter the structure of the molecule. In addition, solubilization of proteins often decreases their immunogenicity. Therefore, most monoclonal antibodies to cell surface antigens have been obtained after immunization of mice with whole cells or membrane fractions. In many cases, specific lymphocyte subsets have been injected into mice resulting in panels of monoclonal antibodies. These antibodies have been used to isolate and characterize the antigen that they bound. When mice are immunized with whole cells, antibodies to a large number of different molecules are generated. It is therefore difficult to use the same cells for the screening of specific antibody production by the hybridoma clones.

To circumvent the above-mentioned problem, we have expressed several known human cell surface proteins in Sf9 insect cells and used these insect cells to immunize mice. Since the introduction of PCR technology (Saiki et al., 1985, 1988), it has become relatively simple to clone the cDNAs for proteins whose coding DNA sequence has been published. One can use PCR primers spanning the complete coding region only, and incorporate restriction sites in these primers to facilitate cloning into expression vectors.

It has been shown that human intracellular, secreted, and transmembrane proteins can be expressed at high levels in Sf9 insect cells when inserted under the regulation of the nonessential baculovirus gene for the polyhedrin protein (reviewed by Luckow and Summers, 1988). Since recombinant proteins can be expressed at very high levels on the cell surface of insect cells, a large proportion of the immune response after injection of these cells will be directed against the recombinant protein. We found that only 2 i.p. injections with 5×10^6 Sf9 cells expressing human CD40 or human B7 gave good serum titers against these antigens. Furthermore, the insect

cells themselves did not evoke an immune response cross-reactive with human cells. This enabled us to use EBV-transformed B cells for the screening of specific antibody production by the hybridoma clones, with minimal risk of obtaining false positives. All of the positive primary wells obtained were in fact specific for the antigen that was used for immunization.

Since we used EBV-transformed B cells for the screening of the primary hybridoma supernatants and during the limiting dilution cloning, the specificity of the monoclonal antibodies remained to be demonstrated. Several lines of evidence presented here suggest that we have indeed generated four anti-(CD40) and one anti-(B7) monoclonal antibodies. First, there is positive staining with these antibodies on EBV-transformed B cells, but not on T cell lines. Secondly, the competition experiment shown in Fig. 3 demonstrated that the anti-(CD40) antibodies bound to a soluble form of CD40 but not to a soluble form of B7. Likewise, the anti-(B7) antibody only bound to the soluble B7 molecule. Finally, the staining with the antibodies on tonsillar B cells as reported in Tables II and III is in agreement with what has been published for other anti-(B7) and anti-(CD40) monoclonal antibodies (Yokochi et al., 1982; Paulie et al., 1985; Freedman et al., 1987; Valle et al., 1990). Although we, like Valle et al. (1990), found a smaller increase in the number of tonsillar B cells expressing B7 after stimulation with immobilized anti-(IgM) antibodies than has been reported by Freedman et al. (1987), this could be due to differences in the experimental procedures used.

It should be mentioned that the fusion data described in this paper, in particular the frequency of positive clones in the B7 fusion, is not optimized. It is therefore difficult to draw conclusions about the efficiency of obtaining specific hybridoma clones using this system. Both fusions were the first and, so far, the only ones that we performed with the mice that were immunized, yet both fusions yielded specific monoclonal antibodies. We therefore feel that the method for the generation of monoclonal antibodies to cell surface antigens described in this paper is relatively fast and convenient.

TOTAL P. 11

23

Acknowledgements

We would like to thank Dr. David Ring for critical review of this manuscript and John Reeder for purification of antibodies.

References

- Chirgwin, J.M., Przybyla, E.A., MacDonald, R.J. and Rutter, W.J. (1979) Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* 18, 5294.
- Clark, E.A. and Ledbetter, J.A. (1986) Activation of human B cells through two distinct cell surface differentiation antigens Bp35 and Bp50. *Proc. Natl. Acad. Sci. USA* 83, 4494.
- De Boer, M., Ten Voorde, G.H.J., Ossendorp, P.A., Van Duje, O., and Tager, J.M. (1988) Requirements for the generation of memory B cells in vivo and their subsequent activation in vitro for the production of antigen-specific hybridomas. *J. Immunol. Methods* 113, 143.
- De Groot, C., Bruhn, J., Mevius, M.L.C.M. and Wormmeester, J. (1990) Contrasting effects of interleukin-4 on the in vitro proliferation of human B cells is due to the presence of B cell subsets in different activation sites. *Lymphokine Rev.* 9, 321.
- DiSanto, J.P., Terry, L.A. and Flomenberg, N. (1991) Generation of anti-human CD8 β -specific antibodies using transfectants expressing mixed-species CD8 heterodimers. *J. Immunol. Methods* 141, 123.
- Freedman, A.S., Freeman, G., Horowitz, J.C., Daley, J. and Nadler, L.M. (1987) B7, a B cell-restricted antigen that identifies preactivated B cells. *J. Immunol.* 139, 3269.
- Freeman, G.J., Freedman, A.S., Segall, J.M., Lee, G., Whitman, J.P. and Nadler, L.M. (1989) B7, a new member of the Ig superfamily with unique expression on activated and neoplastic B cells. *J. Immunol.* 143, 2714.
- Grossenmyer, T., Scheldmann, K.H., Hutchinson, M.A. and Walker, G. (1985) Complexes of polyoma virus medium T antigen and cellular proteins. *Proc. Natl. Acad. Sci. USA* 82, 7932.
- Köhler, G. and Milstein, C. (1975) Continuous culture of fused cells secreting antibodies of predefined specificity. *Nature* 256, 495.
- Luckow, V.A. and Summers, M.D. (1988) Trends in the development of baculovirus expression vectors. *Bio/Technology* 6, 47.
- Malirelli, B., Inlow, D., Shauger, A. and Harano, D. (1988) Large scale insect cell culture for recombinant protein production. *Bio/Technology* 6, 1406.
- Paulle, S., Ehlin-Henriksson, B., Mellstedt, H., Kohn, H., Ben-Alasa, H. and Perlmann, P. (1985) A p50 surface antigen restricted to human urinary bladder carcinomas and B lymphocytes. *Cancer Immunol. Immunother.* 20, 23.
- Saiki, R.K., Scharf, S., Faloona, F., Mullis, K.B., Horn, G.T., Erlich, H.A. and Arnheim, N. (1985) Enzymatic amplification of β -globulin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* 230, 1350.
- Saiki, R.K., Gelfand, D.H., Stoffek, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B. and Erlich, H.A. (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 239, 487.
- Smith, G.E., Ju, G., Erickson, B.L., Muschler, J., Lahm, H.W., Chizzonite, R. and Summers, M.D. (1985) Modification and secretion of human interleukin 2 produced in insect cells by a baculovirus expression vector. *Proc. Natl. Acad. Sci. USA* 82, 8404.
- Stamenkovic, I., Clark, E.A. and Seed, B. (1989) A B cell activation molecule related to the nerve growth factor receptor and induced by cytokines in carcinomas. *EMBO J.* 8, 1403.
- Summers, M.D. and Smith, G.E. (1987) A manual of methods for baculovirus vectors and insect cell culture procedures. Texas Agricultural Experiment Station Bulletin no. 1555.
- Valle, A., Garrone, P., Yssel, H., Bonnefoy, J.Y., Freedman, A.S., Freeman, G., Nadler, L.M. and Banchereau, J. (1990) mAb 104, a new monoclonal antibody, recognizes the B7 antigen that is expressed on activated B cells and HTLV-1-transformed T cells. *Immunology* 69, 331.
- Webb, N.R. and Summers, M.D. (1990) Expression of proteins using recombinant baculoviruses. *Technique* 2, 173.
- Webb, N.R., Madoulet, C., Tosi, P-F., Brunsard, D.R., Snod, L., Nicolau, C. and Summers, M.D. (1989) Cell surface expression and purification of human CD4 produced in baculovirus-infected insect cells. *Proc. Natl. Acad. Sci. USA* 86, 7731.
- Yokochi, T., Holly, R.D. and Clark, E.A. (1982) B lymphoblast antigen (BB-1) expressed on Epstein-Barr virus-activated B cell blasts, B lymphoblastoid cell lines and Burkitt's lymphomas. *J. Immunol.* 128, 823.